

Induction of Apoptosis in Oral Cancer Cells by an Anti-*bcl-2* Ribozyme Delivered by an Adenovirus Vector¹

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ABSTRACT

Human oral cancer cells may have any of several genetic changes, but the role of the *bcl-2* oncogene is relatively unexplored. To find out if this gene plays a significant role and whether it could act as a target for gene therapy of oral cancer, we have examined the effects of an anti-*bcl-2* ribozyme on the phenotype of oral cancer cells. A hammerhead ribozyme was designed to cleave the *bcl-2* transcript after nucleotide 279 and was confirmed to be effective against a synthetic *bcl-2* transcript. A gene encoding the ribozyme was cloned into an adenovirus vector and transferred to the human oral cancer cell lines 686LN, 1483, and Tu183. Over a 6-day period, the growth of each cancer cell line was reduced, whereas growth of the fibroblast cell line FS7 was less inhibited. Inhibition of the oral cancer cells could be attributed to apoptosis, as indicated by the detection of histone-associated DNA fragments in an immunoassay. Northern blots showed no detectable reduction in the level of *bcl-2* mRNA of Tu183 cells, but Western blots showed a reduction of Bcl-2 protein at 24 h after infection with the ribozyme-expressing adenovirus vector. The results imply that (a) expression of the *bcl-2* oncogene is necessary for the survival of oral cancer cells, (b) the *bcl-2* gene transcript presents a target for gene therapy by ribozymes, and (c) an adenovirus vector is a suitable method for transfection of the ribozyme-expressing gene.

INTRODUCTION

Human oral cancers show a variety of genetic changes, with different changes in different tumors. Numerous studies have reported mutation and overexpression of the gene for P53 (1), overexpression of the receptor for epidermal growth factor (2), and the presence of human papillomavirus DNA (3).

Another phenotypic change in oral cancer, which has been

studied less intensively, is overexpression of the oncogene *bcl-2*. The proportion of oral cancers in which Bcl-2 can be seen by immunohistochemical staining has been reported at between 50% and 75% of cases in most studies (4-7), although others have found it only rarely (8, 9). When present, it is generally more prevalent in the poorly differentiated tumors (4-6). In normal oral mucosa, Bcl-2 is not detectable (9) or is expressed only occasionally in the basal cells (10). Bcl-2 is frequently seen adjacent to oral cancers (4, 6) and in dysplastic epithelium (6). There have been no functional studies that explain the role of the Bcl-2 protein in oral cancer, although one study has shown that when oral keratinocytes were transfected with a *bcl-2*-expression plasmid, their level of differentiation markers was reduced (11).

Overexpression of Bcl-2 is common in non-oral cancers. In follicular lymphomas, the overexpression is due to a chromosomal translocation, which places the *bcl-2* gene under the control of a more active promoter (12). Overexpression also occurs in some breast cancers (13), glioblastomas (14), lung cancers (15), and colorectal carcinomas (16). In these tumors, as in oral cancer, the reason for overexpression is unknown because there is no genetic rearrangement. Overexpression of *bcl-2* is not a universal feature of cancer because it is not seen in malignant melanomas (17) or medulloblastomas (14).

The function of the Bcl-2 protein is to block apoptosis in many cell pathways (18), but not all. It cannot block the apoptosis that eliminates autoreactive T cells (19, 20) or apoptosis of lymphocytes induced by the CD95 "death receptor" pathway (21, 22). The Bcl-2 protein does not transform cells by itself, but renders them transformable by other oncogenes such as *myc* (23).

When expression of *bcl-2* is inhibited, some cancer cells lose their malignant behavior. In lymphoma cells, the expression of *bcl-2* can be blocked by antisense RNA, and this modifies the malignant phenotype of the cells (23, 24). An anti-*bcl-2* ribozyme can cancel the ability of Bcl-2 to block the effects of proapoptotic agents on prostate cancer cells that had previously been transduced with *bcl-2* (25). Recently we showed that anti-*bcl-2* oligonucleotides could induce apoptosis in human glioblastoma cell lines.³ In the present study, we have attempted to show whether Bcl-2 is important in the growth of oral cancer cell lines by blocking its function with a specific ribozyme.

MATERIALS AND METHODS

Ribozyme. A hammerhead ribozyme was designed to cleave the transcript of the *bcl-2* gene between nucleotides 279 and 280. The ribozyme gene was then synthesized in two forms.

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65° for 16 h with a probe. The probe had been transcribed with T3 polymerase from the plasmid SYNBL2SM, which contains the *bcl-2* open reading frame (kindly provided by Dr. S. Korsmeyer) and which had been linearized with *Nsp1*. The Ambion MAXIScript *in vitro* transcription kit was used, and labeling was accomplished with T7 polymerase and 32 P dCTP. The membrane was washed to high stringency as described in the directions for the Ambion NorthernMax kit, was exposed to a Molecular Dynamics phosphorimager cassette, and was examined by phosphorimager analysis.

For detection of actin RNA, the membrane was then rehybridized again for 1 h at 65°. An RNA probe for actin was prepared by transcription from the pTRI-ACTIN HUMAN template (Ambion) and labeled as before. This probe was allowed to hybridize for 16 h, washed to high stringency, exposed again for 1 h to a phosphorimager cassette, and examined by phosphorimager analysis.

Expression of Bcl-2 Protein in Cells. To examine the expression of the Bcl-2 protein, Western blotting was performed. Cells were extracted with PBSTDS (Calbiochem-Novabiochem, San Diego, CA), which includes a cocktail of protease inhibitors. As a positive control, the lymphoma cell line SUHL-6, which expresses high levels of Bcl-2 (31), was included as well as several concentrations of a recombinant Bcl-2 protein (PharMingen, San Diego, CA). The concentration of Bcl-2 in the recombinant standard was assessed by its electrophoresis on a 12% polyacrylamide gel alongside several concentrations of albumin, followed by staining of the gel with amido black, and interpolation of the standard curve. In this way, we estimated that the concentration of Bcl-2 in the preparation was 30 ng/ μ l. The total protein concentration of each cell sample was determined by the BCA Protein Assay (Pierce, Rockford, IL), with optical absorbances read at 562 nm. Samples were separated on 12% SDS-PAGE gels, which were loaded with 10 μ g of protein/lane, for 3 h. Proteins were then transferred to a nitrocellulose membrane of 0.45- μ pore size (Bio-Rad, Hercules CA) on a semiautomatic electrotransfer unit (Owl Separation Systems, Woburn, MA). Hamster monoclonal antibody to human Bcl-2 (6C8, PharMingen) was incubated with the blot for 2 h at a 1/100 dilution. Biotinylated secondary antibody was reacted with the Vectastain avidin-biotin complex system and stained with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate using the Vectastain avidin-biotin complex-alkaline phosphatase system (Vector Laboratories, Burlingame, CA). Blots were then scanned by a digital scanner, and the scans were examined by computerized densitometry. A standard curve relating the density of each band to the concentration of Bcl-2 was prepared with the use of several dilutions of the recombinant Bcl-2 protein. Parallel gels were blotted and stained with an antiactin antibody (Sigma Chemical Company, St. Louis, MO), and other lanes of these gels were loaded with different concentrations of bovine actin to act as concentration standards.

Apoptosis. To confirm that the anti-*bcl-2*-ribozyme induced apoptosis in cancer cells and to determine whether the effect of the ribozyme was specific for cancer cells, FS7 cells and Tui83 cells were grown in 96-well dishes that were seeded with 2×10^3 cells/well. After 24 h, the cells were infected with either Av1R279 or with Av1LacZ4, in volumes of 50 μ l/well at titers of either 10^7 or 10^5 pfu/ml. Some cultures were exposed

to camptothecin at 4 μ g/ml. Infected cells were harvested after a further 24 h or 6 days, whereas the camptothecin-exposed cells were harvested after 4 h. These time points and concentrations had been determined to be optimal in pilot experiments.

For harvesting of cells, the supernatants were removed and discarded. The cells were then lysed in buffer and added to microtiter plates coated with streptavidin (Boehringer Mannheim Cell Death Detection ELISA-Plus kit). A mixture of anti-histone-biotin and anti-DNA-peroxidase were then added and incubated for 2 h according to the manufacturer's directions. Nucleosomes were then quantitated by the addition of the substrate, [2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)], and the optical absorbance (A_{405} nm) was determined in a spectrophotometer. Each assay included a DNA-histone complex that was supplied with the kit as a positive control, and a negative control, which consisted of an incubation buffer in place of the cell lysate.

Growth of Cells. To find the effect of the anti-*bcl-2* ribozyme on viability of cells, their growth was monitored by their ability to metabolize (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to its blue product, formazan (32, 33) as before (34). Cells were plated in 96-well plates at a starting number of 1000 cells/well, and some wells were infected the following day with 50 μ l of virus. Cultures were evaluated on the day of infection (Day 0) and on Days 2, 4, and 6. The $A_{560-590}$ value was used as a measure of cell number. Each experiment was performed with at least 6 wells/group/experiment, and each experiment was performed at least twice. For infection by adenovirus, the concentration of virus varied from 10^5 to 10^7 pfu/ml.

RESULTS

Activity of Ribozyme *In Vitro*. The target *bcl-2* sequences were readily cleaved by exposure to the ribozyme, with increasing efficiency up to a ratio of 10:1 and increasing time up to 60 min. At a ratio of 1:1, >50% of the target was cleaved in 1 h (Fig. 2). The calculated value of the K_{cat}/K_m ratio was 2.2×10^{-6} nM/liter/min.

Infection of Cells. The proportion of cells that could be transduced by an adenoviral vector was determined by examining cells that had been infected with Av1LacZ4. With increasing concentrations of virus, increasing proportions of each cell type showed expression of β -gal (Table 1). To obtain staining of 50% of cells, the FS7 and 686LN cells required 10^7 pfu/ml, the 1483 cells required 10^6 pfu/ml, and the Tui83 cells required 10^5 pfu/ml.

The expression of β -gal from infected Tui83 cells was detectable at 12 h after infection with 10^5 and 10^7 pfu/ml of virus and at 18 h after infection with 10^6 pfu/ml. The highest levels of expression were seen with 10^7 pfu/ml at 24 h, 36 h, and 48 h after infection.

Expression of Ribozyme in Cells. The reverse transcription-PCR reaction revealed an amplicon of the expected size in cells that had been infected with Av1LacZ4 or Av1R279, but not in mock-infected cells. The band was cleaved by the restriction enzymes into two fragments of the expected sizes (not shown). This confirmed that the anti-*bcl-2* ribozyme was expressed from the adenoviral vector.

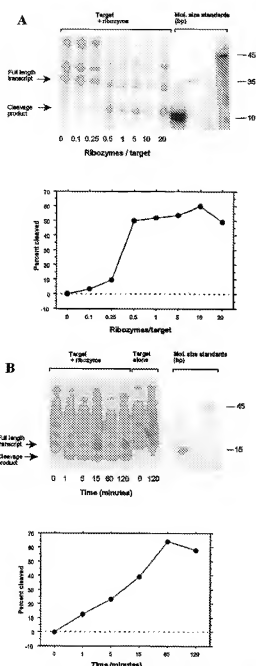


Fig. 2. *In vitro* assays showing the effects of the ribozyme target ratio and of incubation time on cleavage of a synthetic *bcl-2* 19-bp target by the anti-*bcl-2* ribozyme. The upper half of each panel shows a phosphorimage of a gel, and the lower half shows the percentage of the target that is present as a cleavage product. *A*, effects of the ribozyme target ratio when the target and ribozyme were mixed for 60 min. *B*, effects of incubation time when the ribozyme target ratio was 20:1.

Effect of Ribozyme on Expression of *bcl-2* mRNA in Cells. No differences were detected in the level of *bcl-2* mRNA in Tu183 cells that had been infected with either AvIRz279 or the control vectors AvILacZ4 or AvIRz309 (Fig. 3).

Table 1. Proportion of each cell type that expressed β -gal after infection with AvILacZ4

Cells	Virus concentration (p.f.u./ml)			
	10^5	10^6	10^7	10^8
FS7	28% ^a	22%	50%	75%
686LN	10%	25%	85%	100%
1483	10%	75%	100%	100%
Tu183	63%	98%	100%	100%

^a Proportion of cells that stained blue with X-gal at 24 h after infection.

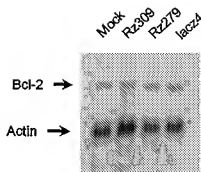


Fig. 3. Northern blot showing the expression of *bcl-2* RNA in cells Tu183. Cells were either mock-infected or infected with one of the adenovirus vectors as indicated. mRNA was extracted after 24 h and blotted to a membrane. The blot was probed first with a probe for *bcl-2* mRNA and then for actin. The figure is a scan from a phosphorimage.

Effect of Ribozyme on Levels of Bcl-2 Protein in Cells. The Bcl-2 protein was readily detected in the control SUDHL-6 cells by Western blotting, as expected (31), and the concentration in different experiments varied between 0.5 μ g and 1.1 μ g/10 μ g of cell protein. In Tu183 cells, the level of Bcl-2 varied between 0.1 μ g and 0.6 μ g/10 μ g of cell protein. In FS7 cells, Bcl-2 was not detectable and was therefore present at a lower concentration than the lowest concentration of the standard, which was equivalent to 0.03 μ g/10 μ g of cell protein (Fig. 4). In 686LN cells and 1483 cells, there were detectable amounts of Bcl-2, but the apparent concentrations were lower than in SUDHL-6 or Tu183 cells, and the bands were not detected in every preparation.

When Tu183 cells were infected with the adenovirus vectors, the level of Bcl-2 was unchanged during the first 6 h (Fig. 4A). After 24 h, the level of Bcl-2 declined in the cells that had been infected with AvIRz279 but remained the same in cells that had been infected with AvILacZ4. In the representative experiment shown in Fig. 4B, the level of Bcl-2 was 0.9 μ g/10 μ g in the presence of AvILacZ4 and 0.15 μ g/10 μ g in the presence of AvIRz279. This was a reduction of 75%. The level of actin was unchanged.

Apoptosis. After 2 days, the mock-infected Tu183 cells showed an A_{490} value of 0.02, and FS7 cells showed a value of 0.006. After 6 days, this had risen to 0.22 for Tu183 cells and 0.024 for FS7 cells. These values were considered as representing background levels and were subtracted from the values obtained from virus-infected cells at the same time points.

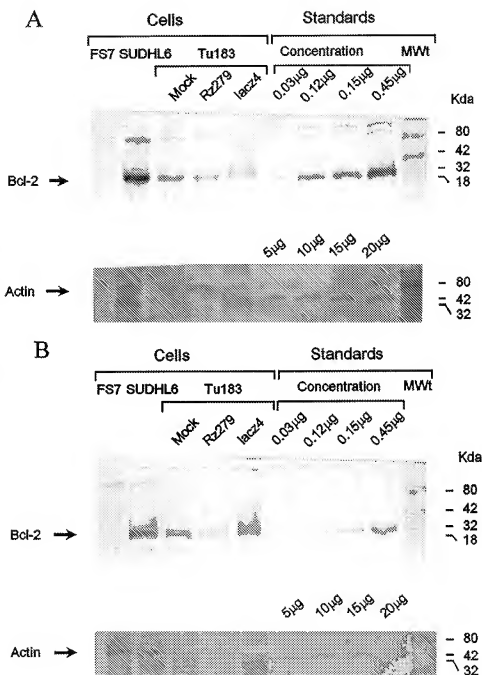


Fig. 4 Western blots showing expression of Bcl-2 protein in FS-7, SUDHL-6, and Tu183 cells. The Tu183 cells were either mock-infected or infected with Av1Rz279 or Av1LacZ4 Adenovirus vectors were at a concentration of 10^7 pfu/ml. In the upper half of each panel, some lanes contained recombinant Bcl-2 standards at the concentrations shown. The upper half of each panel was stained with anti-Bcl-2 serum. In the lower half of each panel, actin was used as a standard, and that half was stained with antiactin serum. A, cells that were infected for 6 h. B, cells that were infected for 24 h.

After 24 h of infection by Av1Rz279, the Tu183 cells showed apoptosis as indicated by a corrected A_{425} value of 0.49 when a virus titer of 10^5 pfu/ml was used. This dropped to 0.1 after 6 days (Fig. 5). When lower concentrations of virus were used, apoptosis was induced with peak values at later times (data not shown). Av1LacZ4 did not induce apoptosis in Tu183 cells at any time tested at either of the time points used, and neither Av1Rz279 nor Av1LacZ4 induced apoptosis in FS7 cells (Fig. 5). Camptothecin induced apoptosis in Tu183 cells but with a maximum corrected absorbance value of only 0.031.

Growth of Cells. When cells were infected with the concentration of adenovirus that transduced 50% of cells, each of the oral cancer cell lines was inhibited by Av1Rz279 but not by Av1LacZ4. FS-7 cells were not inhibited under these conditions by either of the adenovirus vectors (Fig. 6). When the concentration of the virus vectors was varied from 10^5 up to 10^8 pfu/ml and growth inhibition was expressed as a percentage reduction on the sixth day of growth, some toxicity was detectable with each virus vector (Fig. 7); under these conditions, Av1Rz279 produced up to 48% inhibition of FS7 cells and up to

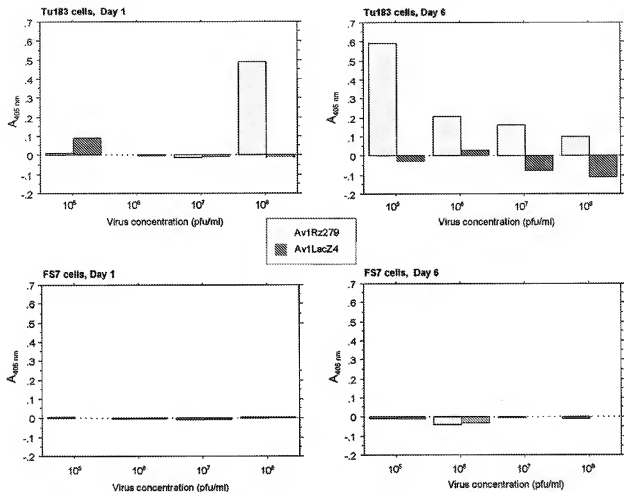


Fig. 3. Apoptosis after infection by either Av1Rz279 or Av1LacZ4. Cells were Tu183 (upper panels) or FS7 (lower panels). Apoptosis was detected by an ELISA technique to detect histone-bound DNA. The corresponding value from mock-infected cells was subtracted.

98% inhibition of Tu183 cells. Av1Rz279 was always more inhibitory than Av1LacZ4 by about two logarithmic dilutions.

DISCUSSION

A role for the Bcl-2 protein in oral cancer has often been suggested because of its visibility in some cases after immunohistochemical staining. However, its role in this disease has yet to be established. In the present study, the Bcl-2 protein was detectable in both the G86LN and 1483 oral cancer cells. In Tu183 oral cancer cells, the level of expression of Bcl-2 was extremely high and was similar to that of the lymphoma cell line SUDHL6 (Fig. 4). In contrast, the protein could not be detected in the FS7 fibroblasts. These data support a role for Bcl-2 in at least some oral cancers.

When a short synthetic fragment of the *bcl-2* transcript was exposed to a ribozyme, it was found that the transcript had a susceptible site for cleavage between nucleotide 279 and 280. The site was found to be extremely sensitive, with >50% of the target cleaved within 1 min (Fig. 2,4). These data suggested that a ribozyme might be used for examining the role of *bcl-2* in oral

cancer and exploring new therapeutic options. A gene that encoded the ribozyme could be introduced into cells quite readily by the use of an adenovirus vector. However, it was found that the different cells used in this study were transfected at different efficiencies by the adenovirus, and multiple concentrations of adenovirus were therefore used for evaluation of the ribozyme.

When the ribozyme was expressed in Tu183 cells, it led to a marked reduction in the level of the Bcl-2 protein (Fig. 4), which is consistent with its potent anti-Bcl-2 effect. Interestingly, the ribozyme did not produce a detectable reduction in the level of the *bcl-2* mRNA in the cells (Fig. 3). Therefore, although the ribozyme does have catalytic activity, the reduction of the Bcl-2 protein might have been due at least in part to the antisense activity of the flanking arms. It is known that *bcl-2* can be inhibited by antisense oligonucleotides in lymphoma, glioma, and melanoma cells and that this affects the malignant phenotype and induces chemosensitivity (23, 35, 36).⁷ Thus, the relative contributions of antisense and catalytic activities of Rz279 remain to be determined.

The reduction of the level of the Bcl-2 protein was accom-

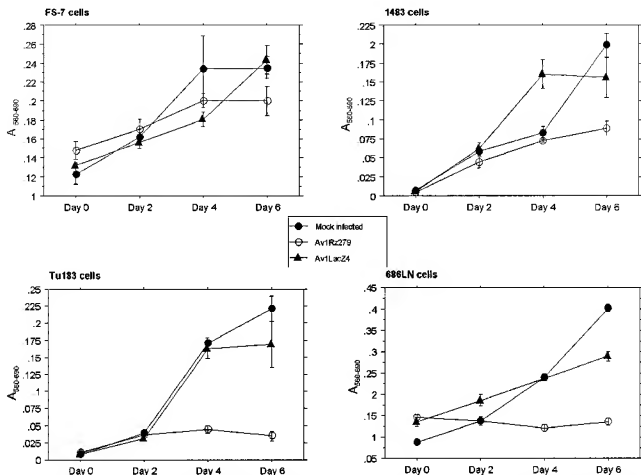


Fig. 6 Growth curves of cells after either mock-infection or infection with Av1Rz279 or Av1LacZ4. Each cell line was infected with the titer of virus that transduced 50% of cells. This was 10^7 pfu/ml for FS-7 and 696LN cells, 10^6 for 1483 cells, and 10^5 for Tu183 cells. Growth was monitored by the (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay.

panied by the appearance of apoptosis in the Tu183 cells, as shown by the appearance of histone-bound fragments of DNA (Fig. 5). Apoptosis appeared more rapidly when higher concentrations of virus were used, as might be expected because the marker gene was also expressed sooner with higher levels of virus. By 6 days after infection with Av1Rz279, apoptosis was detected with even the lowest concentration of virus, although by this time, the levels from the higher concentrations had passed their peak and were declining. FS7 cells did not show apoptosis with any concentration of virus at either of the two time points studied, which is consistent with the lack of Bcl-2 protein in these cells.

Consistent with the induction of apoptosis, the expression of the ribozyme affected the growth of the oral cancer cells with less effects on fibroblasts (Figs. 6 and 7). The ribozyme was particularly effective on the Tu183 cells, which are the cells that expressed the highest level of Bcl-2. Interestingly, the MMTV promoter that was used to express the ribozyme was found recently to be relatively weak in oral cancer cells (37). Thus, even stronger anti-bcl-2 effects could be expected if a stronger promoter was used.

Adenovirus vectors do have some nonspecific inhibitory effects on oral cancer cells, as we have noted before (38). The nonspecific toxicity is not related to the multiplicity of infection, but rather to the concentration of virus³, and thus we have not shown the data in terms of multiplicity of infection. The nonspecific effects were easily distinguished from specific effects in four ways: (a) The anti-bcl-2 ribozyme was more inhibitory in cells that expressed the Bcl-2 protein. (b) At the virus concentration, which transduced 50% of cells, the ribozyme-expressing virus was more toxic than the β -gal-expressing virus in each cell type. (c) The ribozyme-expressing virus induced apoptosis in the Tu183 cells but not in fibroblasts, and the β -gal-expressing virus did not induce apoptosis in either cell type. (d) The ribozyme-expressing virus reduced the level of the Bcl-2 protein in Tu183 cells, whereas the β -gal-expressing virus did not.

The present study appears to be the first that shows the use of a ribozyme to modify the transformed phenotype of a tumor

³ Gibson *et al.*, manuscript in preparation

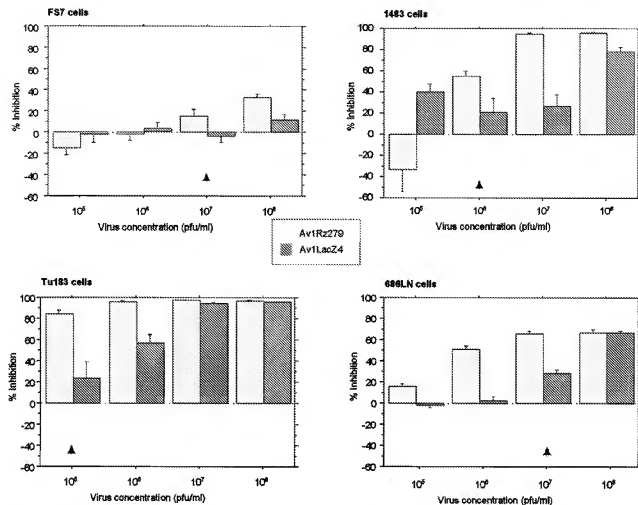


Fig. 7. Percent inhibition of growth of each cell type by different concentrations of adenovirus vectors after 6 days of culture under the conditions indicated for Fig. 6. The arrows on the X-axes indicate the virus titer at which 50% of the cells were transduced.

cell line that endogenously expresses *bcl-2*. In an earlier study, an anti-*bcl-2* ribozyme did affect the phenotype of a prostate cancer cell line, but the cells had previously been transfected with a *bcl-2*-expressing plasmid (25). Furthermore, the target site that was used in the present study, nucleotide 279, does not appear to have been used previously in either antisense or ribozyme studies.

The data from this study suggest that both pro- and anti-apoptotic mechanisms are at play in oral cancer cells. The induction of apoptosis by the anti-*bcl-2* ribozyme implies that these cells are constitutively expressing a proapoptosis phenotype, which is simultaneously being suppressed by Bcl-2. The origin of the proapoptosis phenotype is unknown, although there are at least three possibilities: (a) Normal epithelial cells are programmed to differentiate, undergo apoptosis and die, and this mechanism might persist in oral cancer. (b) Oral cancer occurs in patients whose cells are inherently susceptible to DNA damage by mutagens (39), and DNA damage generally leads to apoptosis (18). (c) When epithelial cells are separated from their substrate, this triggers apoptosis through a mechanism known as

anoikis (40), which can be prevented by overexpression of Bcl-2 (41). The regulation of apoptosis in oral cancer might involve many other proteins apart from Bcl-2. A large number of pro- and antiapoptotic proteins have been documented in various cell types (18) and could be involved in the regulation of apoptosis in oral epithelium and oral cancer. However, nothing appears to be known about the regulation of apoptosis in oral cancer except that the present data imply that it is a Bcl-2-sensitive pathway that produces the antiapoptotic signal.

If expression of an anti-*bcl-2* ribozyme can inhibit the growth of oral cancer cells, this might be useful in cancer therapy. The fact that the ribozyme can be delivered by a viral vector suggests that this is possible. The fact that the fibroblast line FS7 was not inhibited implies that this could be relatively safe. A further advantage of any anti-*bcl-2* treatment would be that it might sensitize cells to conventional therapy because Bcl-2 is known to inhibit the anticancer effects of several chemotherapeutic drugs (42, 36). It has been shown that levels of Bcl-2 can be reduced by retinoic acid with resulting sensitivity to chemotherapy (43), and it is known that retinoic acid

can reduce the malignant progression of leukoplakia and inhibit development of second primary and recurrent oral cancers (44). It is thus possible that the protective effects of retinoids occur by their effect on a proapoptotic pathway that involves Bcl-2.

There are other examples of ribozymes that inhibit the malignant phenotype of human cancer cells when delivered by a viral vector. Adenoviruses have been used to deliver ribozymes against *ras* to bladder and lung cancer cells (45) and against HER-2/*neu* or pleiotropin to other cancer cells (46). Retroviruses have been used to deliver ribozymes against *ras* to malignant melanoma cells (47) and against BCRABL to lymphoid cells (48). Despite these few examples, most earlier studies of ribozyme expression have used a plasmid expression vector or used target cells that were transfected with the transforming gene. Examples of targets include papillomaviruses (30, 49), EBV (50), epidermal growth factor (51), and *ras* (52, 53). The present study extends the potential use of virus vectors for the expression of ribozymes in human cancer cells and increases the spectrum of potential targets in oral cancer to include the *bcl-2* oncogene.

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